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# Hormone-inducible casein messenger RNA in a serum-free organ culture of whole mammary gland

(indirect radioimmunoassay/ascites ribosome protein synthesis system/radioimmunoprecipitation/sodium dodecyl sulfate-polyacrylamide gels)

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ABSTRACT **The whole second thoracic mammary gland of estradiol-17β + progesterone primed 3- to 4-week-old BALB/c female mice was induced to pregnancy-like lobulo-alveolar morphogenesis after 6-day cultivation in a serum-free culture medium containing a "growth promoting" hormone mixture, insulin + prolactin + growth hormone (somatotropin) + estradiol + progesterone. No radioimmunologically detectable casein was present in these glands. Subsequent cultivation for another 6 days in a "lactogenic" medium with the hormones insulin + prolactin + cortisol produced abundant milk-like secretory material in the alveolar lumen. RNA of the mammary gland after estradiol + progesterone priming or cultivation in the "growth-promoting" medium failed to show a measurable amount of casein mRNA activity when assayed in a cell-free protein synthesis system derived from Ehrlich ascites ribosomes, rabbit reticulocyte factors, and tRNA. However, the glands sequentially cultivated in the "growth-promoting" and the "lactogenic" media showed a high level of casein mRNA activity in the heterologous cell-free protein synthesis system. Sodium dodecyl sulfate/polyacrylamide gel electrophoretic characteristics of the immunoprecipitable (by antibody to mouse milk casein) polypeptides directed by the mammary RNA induced in organ culture medium containing the lactogenic hormones were similar to the characteristics of the polypeptides directed by mammary polysomes of lactating mice. These results demonstrate hormonal induction of a specific mRNA in a sequential two-step culture of an entire organ in a serum-free chemically defined medium.**

Studies on the mechanisms of hormone action regulating growth and differentiation are generally done in endocrinectomized and/or exogenous hormone-treated animals. But systemic complexities often limit interpretation of the results concerning interactions between the hormone(s) and the target cells. Consequently, the need for appropriate culture conditions that are conducive to specific hormone-responsive physiological events in the target tissue, including the chick oviduct, has been emphasized (1, 2). Ichinose and Nandi (3) have demonstrated that pregnancy-like lobulo-alveolar morphogenesis of the immature mammary parenchyma can be obtained in a culture medium by cultivating the whole mammary gland of the mouse in presence of appropriate "growth-promoting" hormones. We have shown that the immature mammary gland initially induced to lobulo-alveolar morphogenesis in medium with the "growth-promoting" hormones insulin (I) + prolactin (Prl) + growth hormone (GH, somatropin) + estradiol-17β + progesterone + aldosterone undergoes functional differentiation (including production of radioimmunologically detectable casein) after cultivation with the lactogenic hormone combination I + Prl + cortisol (4).

Mammary cells responding to appropriate hormones in a serum-free, chemically defined medium thus provide a favorable system for studying the multiple hormone interactions regulating production of the milk protein. The specific protein synthesis associated with most endocrine-dependent systems is believed to be regulated by hormonal modification of mRNA population in the target tissue (5, 6). Testing the biological properties of hormone-inducible mRNA requires a direct assay system. Previously we have reported that casein mRNA of the lactating mammary gland of the mouse is faithfully translated in a heterologous cell-free protein synthesis system, derived from ascites tumor cell ribosomes, rabbit reticulocyte factors, and tRNA (7). This report presents the results of our studies on cell-free translation of casein mRNA induced by lactogenic hormones in a serum-free organ culture of the whole mammary gland obtained from an immature female mouse.

Abbreviations: E, estradiol-17β; P, progesterone; I, insulin; Prl, prolactin; GH, growth hormone (somatotropin).

## MATERIALS AND METHODS

**Organ Culture.** As a prerequisite for the organ culture procedure (3) 3- to 4-week-old BALB/c female mice were primed for 9 days by daily injections (subcutaneous) of 1 μg of estradiol-17β (E) and 1 mg of progesterone (P) in 0.9% saline suspension. The entire second thoracic mammary gland was excised under sterile conditions and cultivated in Falcon plastic culture dishes (60 × 15 mm) containing chemically defined Waymouth's (8) medium (MB752/1 supplemented with L-glutamine (350 μg/ml), penicilin (35 μg/ml), and different combinations of hormones. The incubation was carried out at 37° in an atmosphere of 95% $O_2$ + 5% $CO_2$ according to the standard procedure (9). The method of organ culture of the whole mammary gland of the mouse has been described in detail (4, 10). For morphological studies the glands were fixed in a mixture of acetic acid and ethanol (1:3) and for biochemical analysis glands were frozen in liquid nitrogen and stored at −80°.

**Indirect Radioimmunoassay.** The mammary tissue was homogenized in a buffer containing 10 mM $NaPO_4$ + 15 mM NaCl (pH = 7.5). The homogenate was centrifuged at 100,000 × *g* for 1 hr at 4°. Different concentrations of protein (11) in the resulting $S_{100}$ supernate were assayed for presence of casein by an indirect radioimmunoassay using $^{125}$I-labeled mouse milk casein and casein antiserum, as previously described (12).

**RNA Extraction.** Frozen mammary tissue was pulverized in a mortar and pestle cooled by liquid nitrogen. The tissue powder was then homogenized in a power-driven glass tissue grinder containing six volumes of buffer A (13) and the RNA was extracted by phenol/chloroform, followed by high-salt wash as described by Palmiter (13). According to Palmiter, for translational assays this procedure yields a high level of biologically active messenger RNA. In the present study the tissue homogenate was shaken with phenol for 40–60 min before addition of chloroform. The RNA was dried by vacuum desiccation at room temperature, and the dehydrated RNA pellet

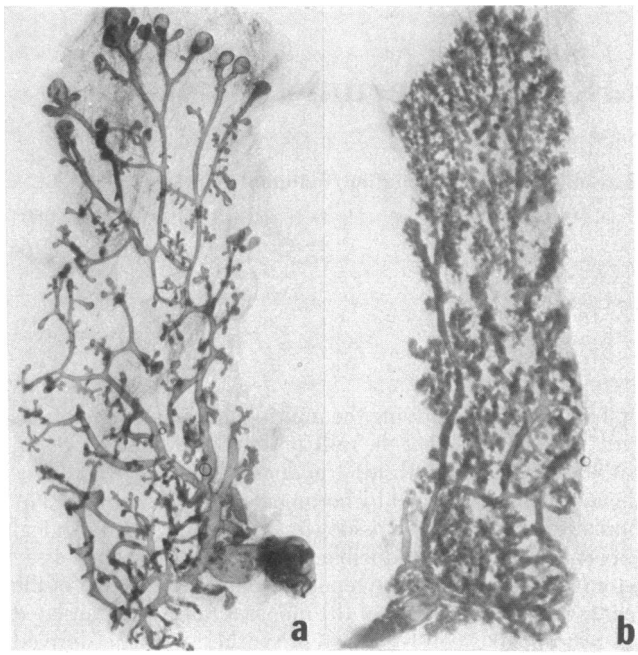


FIG. 1. Stained whole mount preparation of second thoracic mammary gland of BALB/c mouse. (×7.) (*a*) The gland from a 4- to 5-week-old female after daily injections of E (1 μg) and P (1 mg) for 9 days. (*b*) A gland after 6-day cultivation in medium containing I, Prl, GH (5 μg/ml each), E (0.001 μg/ml), and P (1.0 μg/ml).

was dissolved in deionized water and stored at −20° for subsequent assay for casein mRNA activity. For RNA isolation from organ culture glands, 1.55–1.7 g of tissue (86–108 glands) yielded 35–68 $A_{260}$ units of RNA (One $A_{260}$ unit is the amount of material that has an $A_{260}$ of 1 when it is dissolved in 1 ml and the light path is 1 cm.) Two grams of E + P primed tissue yielded 28 $A_{260}$ units of RNA.

**Direct Assay for Casein mRNA.** Casein mRNA activity of the mammary RNA was monitored in a heterologous cell-free protein synthesis system, derived from Ehrlich ascites tumor cell ribosomes ($S_{30}$ fraction), rabbit reticulocyte factors (0.5 M KCl wash of reticulocyte $S_{30}$), and tRNA as previously described (7). Casein in the reaction product of the cell-free protein synthesis system was determined by specific immunoprecipitation, using antibody to mouse milk casein prepared in our laboratory (12). The immunoprecipitable material in the reaction product was further characterized by sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

**Culture Medium, Hormones, and Labeled Compounds.** Ready-to-use Waymouth's medium MB752/1 (8) was supplied by Microbiological Associates, Bethesda, MD. The hormones insulin (bovine), growth hormone (bovine), prolactin (ovine), progesterone, and estradiol-17β were from Calbiochem, La Jolla, CA, and cortisol was from Sigma Chemical Co., St. Louis, MO. Iodine-125 ($^{125}I$), 17 Ci/mg of I (NaI in 0.1 M NaOH), was purchased from ICN, Isotope and Nuclear Division, Irvine, CA. The amino acids [2,3,4,5-$^{3}H$]proline (71.0 Ci/mmol) and [4,5-$^{3}H$]leucine (67.6 Ci/mmol) were supplied by New England Nuclear Corp., Boston, MA.

## RESULTS

**Morphogenesis and Functional Differentiation.** Fig. 1 illustrates that after 6 days of cultivation with "growth-promoting" hormones I + Prl + GH + E + P, the ductal mammary parenchyma of the E + P primed gland developed pregnancy-like lobulo-alveolar structures. These whole mounts illustrate that, like I + Prl + GH + E + P + aldosterone (4), the present medium with I + Prl + GH + E + P also produces pregnancy-like lobulo-alveolar development. No histologically detectable secretory material was present in the gland after cultivation with the "growth-promoting" hormones, whereas milk-like secretory material was abundant in the gland after sequential cultivation in medium containing the "growth-promoting" and the lactogenic hormones I + Prl + cortisol (Fig. 2). The indirect radioimmunoassay with antibody to mouse

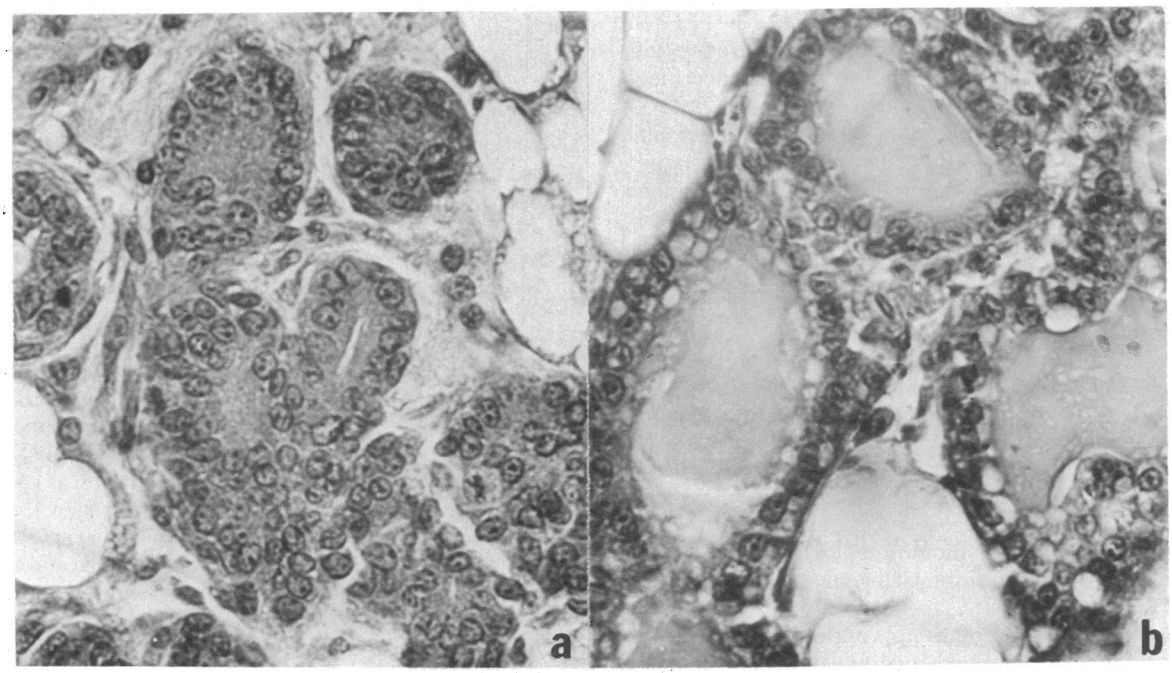


FIG. 2. Histology of glands after cultivation in organ culture. (×400.) (*a*) Six-day cultivation in the medium supplemented with the hormones I + Prl + GH + E + P. (*b*) Sequential cultivation first 6 days in medium with I + Prl + GH + E + P and then another 6 days in medium with I + Prl + cortisol (5 μg/ml). Note abundant milk-like secretory material in gland sequentially cultivated with I + Prl + GH + E + P and I + Prl + cortisol.

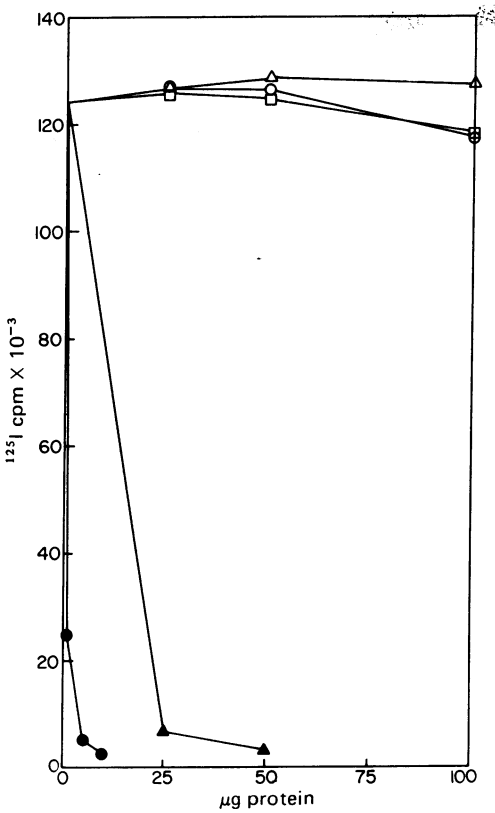


FIG. 3. The results of an indirect radioimmunoassay for mouse casein. Different concentrations (protein) of mammary gland extracts ($S_{100}$), mouse serum, or mouse milk were mixed with 10 μl of casein antiserum, and the solution was incubated for 30 min at room temperature and then overnight at 4°. This was followed by addition of 2.5 μg of $^{125}$I-labeled casein to the solution and the reaction was allowed to continue for 2 hr at room temperature. One milliliter of buffer [0.01 M $NaPO_4$ (pH = 7.5), 0.15 M NaCl, 3% (vol/vol) Triton X-100] was added to the reaction mixture and the content of each tube was mixed thoroughly and passed through 0.45 μm Millipore filter discs (HAWP) pre-wet with the same buffer. The filters were washed with 20 ml of the same buffer and radioactivity trapped on the filter was measured in a well-type gamma scintillation counter (Nuclear Chicago). Data on lactating mammary tissue and milk assayed in the same experiment are included as positive controls and mouse serum as a negative control. For further details see *Materials and Methods.* (△) Mouse serum; (○) 9-day E + P primed; (□) 6-day culture in I + Prl + GH + E + P; (▲) 8-day lactating; (●) mouse milk, prepared by centrifugation of milk at 85 × *g* for 5 min and diluted in 10 mM $NaPO_4$ (pH = 7.5) + 15 mM NaCl [total protein concentrations in milk protein (11) samples were standardized to 50 μg by adding appropriate amounts of bovine serum albumin]. When normal rabbit serum was substituted for casein antiserum in assays of samples of 50 μg of protein from 8-day lactating, 9-day primed, 6-day culture in I + Prl + GH + E + P extracts, or controls with mouse serum or no protein added, the cpm values ranged from 1392 to 1816.

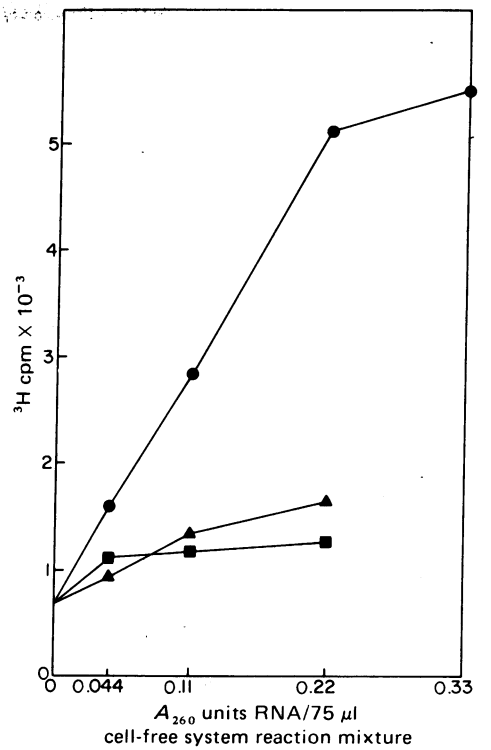


FIG. 4. The results of a direct assay of the mammary gland mRNA in a heterologous cell-free protein synthesis system. The standard assay mixture was as described (7), with the following modifications. Eighteen unlabeled amino acids (56 μM each) and 6.6 μCi/ml each of [$^{3}$H]proline and [$^{3}$H]leucine were used. For specific immunoprecipitation assay using mouse milk antibody, 20 μl of a solution containing 0.05 M each of leucine and proline, 0.01 M $NaPO_4$ (pH = 7.5) and 8% Triton X-100 was added to 50 μl of the cell-free reaction mixture at 0°. Ten microliters (protein concentration 0.1 mg/ml) of mouse milk carrier [prepared by centrifugation at 85 × *g* for 5 min and diluted with 10 mM $NaPO_4$ (pH = 7.5) + 15 mM NaCl] and 20 μl of the casein antiserum were added. The mixture was incubated for 30 min at 25°. One milliliter of "wash" buffer [0.01 M each of leucine and proline, 0.01 M $NaPO_4$ (pH = 7.5), 3% Triton X-100, and 0.15 M NaCl] was added, the solution was thoroughly mixed, and the immunoprecipitate was immediately collected on an 0.45 μm Millipore filter (HAWP) pre-wet with "wash" buffer. The filters were rinsed with 20 ml of the "wash" buffer and assayed for radioactivity by liquid scintillation spectrometry. For further details see *Materials and Methods.* (●) Culture, 6 days I + Prl + GH + E + P followed by 6 days I + Prl + cortisol. Assay of RNA obtained from glands cultivated 6 days in I + Prl + A medium followed by 6 days in I + Prl + cortisol also showed a similar level of casein mRNA activity. (▲) Culture, 6 days I + Prl + GH + E + P; (■) 12 days primed with E + P. Assay of 0.1 $A_{260}$ unit of liver RNA (7) gave 1115 ± 183 (SEM) cpm.

milk casein failed to detect casein in glands after 9-day treatment of the animal with E + P or after 6 days of cultivation of the 9-day primed gland with I + Prl + GH + E + P in organ culture (Fig. 3). Previously we have reported (12) that this assay can detect the presence of casein in glands sequentially cultivated with I + Prl + GH + E + P + aldosterone and I + Prl + cortisol. However, because glands cultured for the initial 5 days in I + Prl + GH + E + P + aldosterone also produced a detectable level of casein (data not shown) the present growth-promoting medium with I + Prl + GH + E + P but no aldosterone was preferred.

**Casein mRNA.** Although mammary glands after 9-day E + P priming were radioimmunologically negative for casein, animals in this group were given 12 days of E + P injections to check whether an extended period of priming may induce casein mRNA production *in vivo*. As shown in Fig. 4, RNA extracted from mammary glands primed *in vivo* with E + P for 12 days and assayed in the heterologous cell-free protein synthesis system showed a negligible ability to direct the synthesis of polypeptides immunoprecipitable by the murine casein antibody (Fig. 4). Lobulo-alveolar morphogenesis of the gland in culture with I + Prl + GH + E + P was similar regardless of 9 or 12 days E + P priming of the animal. A similar negligible level of casein mRNA activity was also evident in RNA extracted from mammary glands cultivated for 6 days in the "growth-promoting" medium containing the hormones I + Prl + GH + E + P (Fig. 4). However, RNA extracted from glands

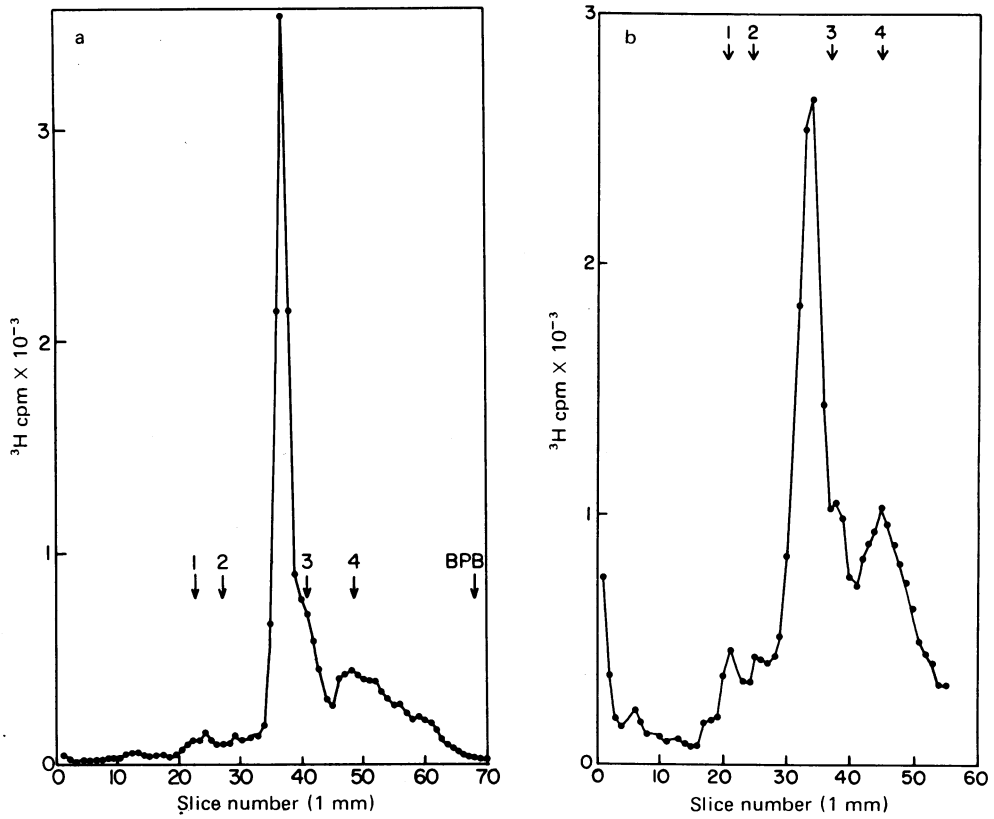


FIG. 5. Sodium dodecyl sulfate/polyacrylamide gel electrophoretic characterization of the material directed by organ culture mammary gland RNA (*a*), and polysomes of lactating mammary cells (*b*). (*a*) Immunoprecipitable products directed by RNA isolated from glands in organ culture 6 days in I + Prl + GH + E + P, followed by 6 days in I + Prl + cortisol medium. After 40-min incubation the ribosomes in the *in vitro* protein synthesis reaction mixture were removed by 105,000 × *g* centrifugation for 1 hr at 4° as described by Rosen *et al.* (14). The reaction product was immunoprecipitated as in Fig. 4 with the modification that the incubation was extended to 1 hr. After addition of "wash" buffer (Fig. 4) and thorough mixing, the sides of the tubes were carefully rinsed with 1 ml of 0.9% saline and the solution was centrifuged 5 min at 10,000 × *g*. The pellet was suspended by addition of 0.25 ml of 1% sodium dodecyl sulfate plus 0.01 M dithiothreitol. The suspension, which had dissolved on standing overnight, was heated 1 min in a boiling-water bath and dialyzed (14). The material was analyzed on a 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate as described (15). (*b*) Polysomes of 5-day lactating mammary gland were assayed for their protein synthesis activity in a previously described reaction mixture (7) with the following modifications. Mammary polysomes, isolated by the procedure described (16), were added (0.415 $A_{260}$ unit/75-$\mu$l reaction mixture) in place of the ascites ribosomes ($S_{30}$). The cell-free reaction mixture was immunoprecipitated and electrophoresed as previously described (7). After electrophoresis the gels were frozen in test tubes lying on crushed dry ice and 1 mm slices were cut with a Mickle gel slicer. The slices were laid in 25 $\mu$l of water in liquid scintillation vials. Ten milliliters of 4% NCS in Omnifluor/toluene was added and the gel slices were allowed to swell overnight at 37° before counting in a Beckman LS 350 liquid scintillation spectrometer. Arrows 1, 2, 3, and 4 indicate positions of milk proteins determined in a parallel gel. BPB, bromophenol blue (also determined in parallel gel).

cultivated first for 6 days in the "growth-promoting" medium, followed by another 6 days with the lactogenic hormone combination, I + Prl + cortisol, demonstrated a high level of specific mRNA activity directing the *in vitro* synthesis of protein precipitable by antibody to mouse casein (Fig. 4).

The immunoprecipitable material of the cell-free reaction product was then characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 5*a*). The radioactive immunoprecipitable proteins whose synthesis was directed by the organ culture mammary RNA migrated in the gel as those directed by the polysomes of mammary glands from lactating mice (Fig. 5*b*). The predominant peak of immunoprecipitable radioactive protein synthesized by the RNA of the organ culture gland and the polysomes of lactating mammary cells migrated in close correspondence with the third peak of the mouse milk protein (Fig. 5 *a* and *b*). The predominant immunoprecipitated product from a cell-free protein synthesis directed by casein mRNA of lactating mammary glands of rats in wheat germ ribosome system has been reported (17) to exhibit a similar profile in sodium dodecyl sulfate polyacrylamide gels. Thus, the casein mRNA induced by lactogenic hormones in the culture medium appears to direct the synthesis of a component of the milk protein that is similar in immunological and molecular weight characteristics to that directed by the RNA from lactating mammary gland *in vivo*.

## DISCUSSION

The virtual absence of casein in the gland after E + P priming *in vivo* or after the first phase of cultivation with the hormones I + Prl + GH + E + P indicates that initial pregnancy-like lobulo-alveolar morphogenesis of the parenchyma in culture is not accompanied by a concurrent functional activity as observed during pregnancy mammary gland *in vivo* (18). Thus, casein found in the gland after the second phase of cultivation is the result of I + Prl + cortisol induced production of the milk protein in the culture medium. Previous studies (19, 20) have established the involvement of I, Prl, and cortisol in regulation of the milk protein, in short-term cultures of fragments of (mostly pregnancy) mammary tissue, and these results have been extensively reviewed (21–23). Some functional activity

in the mammary gland is associated with advancement of pregnancy (18) and recent reports indicate that casein (12) as well as casein mRNA (17) is detectable in mammary tissue of pregnant mice and rats. Thus, production of casein exclusively during the second phase of the present culture system of the whole mammary gland provides an advantage for future studies on the synergism between prolactin and cortisol in regulation of casein synthesis.

The direct assay of mammary RNA in the heterologous cell-free protein synthesis system demonstrates that the specific activity of mRNA coding for casein is virtually nondetectable in glands primed *in vivo* with E + P or after the first phase of cultivation of the glands with the discrete combination of growth-promoting hormones. But a pronounced level of casein mRNA activity, evident in the gland after the second phase of cultivation with I + Prl + cortisol, strongly indicates a *de novo* induction of the mRNA in the culture medium. This finding thus constitutes a direct demonstration of hormone-inducible modification of a specific mRNA population in a target organ during its sequential morphogenetic and functional development in a serum-free, chemically defined culture medium. Molecular homology of the casein mRNA hormonally induced in culture medium with its counterpart *in vivo* is further confirmed by the characteristic similarity of the sodium dodecyl sulfate polyacrylamide gel electrophoresis profile of the predominant immunoprecipitable protein product directed by lactating mammary RNA (polysomal) and the RNA induced in the culture.

Among the hormones, I + Prl + cortisol, needed for production of casein in mammary explants in culture, insulin, a metabolic hormone, is required for maintenance of the epithelium in the culture medium (24, 25). Recent results obtained in our laboratory showed that adrenalectomy-induced 85–92% reduction of casein mRNA activity of the lactating mammary gland of the mouse is preventable by cortisol injections. This strongly indicates a regulatory role of the glucocorticoid on casein mRNA. The same studies further revealed that the specific functional activity of the casein mRNA in total mammary RNA is also influenced by intensity of suckling (unpublished), suggesting that prolactin may influence the cellular concentration of the functional mRNA, because suckling is believed to influence pituitary prolactin release (26). Accordingly, understanding of the interaction between the glucocorticoid and prolactin is important for the elucidation of the molecular mechanism of synergism involved in multiple hormonal induction of casein mRNA. We believe that the present finding of a high level of casein mRNA activity in the whole mammary gland cultivated in medium supplemented with lactogenic hormones but not in medium supplemented with the growth-promoting hormones alone greatly enhances the feasibility of reliable studies concerning hormone-regulated developmental biology of the mammary gland. Recently we have assayed lactating mammary gland (*in vivo*) RNA in a wheat germ ribosome protein synthesis system and the results showed a nearly two-fold greater antiserum-bound activity than what was obtained after translation of the same RNA sample in the ascites ribosome system. This indicates that the wheat-germ ribosome system may prove to be a more sensitive cell-free assay for mouse casein mRNA.

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